

## PLASMID COMPOSITION OF *Shigella flexneri* AND *Shigella sonnei* STRAINS ISOLATED FROM DIARRHOEIC AND ASYMPTOMATIC CHILDREN IN NORTHEASTERN BRAZIL

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### ABSTRACT

Nine *Shigella flexneri* and six *S. sonnei* strains were isolated from asymptomatic (3 *S. flexneri* and 2 *S. sonnei*) and diarrhoeic children in Recife. All strains were compared in respect to invasiveness (Sereny test), colicin production, binding of Congo Red and resistance to antibiotics. The isolates harbored two to seven different plasmids and most had molecular weights smaller than 6 MDal. The *S. sonnei* samples isolated from asymptomatic carriers contained considerably less plasmids than the strains recovered from diarrhoeic patients. The possible association between plasmids and resistance to antibiotics, colicin E1 production and invasiveness is discussed.

### INTRODUCTION

*Shigella* species are among the most important causes of diarrhoeal disease in children throughout the world, representing a serious public health problem in South America and other undeveloped regions (Gordon *et al.*, 1964; Stoll *et al.*, 1982; Guerrant *et al.*, 1983; Keusch and Formal, 1984).

In Brazil, *S. flexneri* is the most prevalent *Shigella* serotype isolated from stools of diarrhoeic children. *S. sonnei* can be also found but in smaller numbers than *S. flexneri* (Pessoa *et al.*, 1978; Teixeira *et al.*, 1984; Giugliano and Giugliano, 1985; Leal *et al.*, 1988a).

Even though considered an obligatory pathogen, *Shigella* can be isolated from carriers without any symptom of bacillary dysentery (Mata *et al.*, 1966; Figueroa *et al.*, 1983). In a previous survey carried out in Northeast Brazil, both *S. flexneri* and *S. sonnei* could be isolated from stools of healthy children from different low income communities (Leal *et al.*, 1988b).

Plasmids are frequently harbored by *Shigella* strains, and they are usually responsible for virulence-associated factors, such as invasion of epithelial cells, intracellular multiplication and spread to adjacent cells (Maurelli and Sansonetti, 1988). Moreover, *Shigella* plasmids are involved in the expression of antibiotic resistance markers and synthesis of colicins (Jamielson *et al.*, 1979; Lee *et al.*, 1982; Mendonza *et al.*, 1988; Bratoeva and John, 1989). Plasmid patterns of different *Shigella* isolates can also be used as an important epidemiological tool to differentiate strains in a community with shigellosis (Tacket *et al.*, 1984).

In the present study, an initial search for phenotypic and genetic differences among *S. flexneri* and *S. sonnei* strains isolated from diarrhoeic and asymptomatic carriers was performed. Drug resistance markers, colicin production, invasibility, adsorption of pigments, and plasmid content were investigated. The possible involvement of these characteristics with the virulence of the isolates is discussed.

## MATERIALS AND METHODS

### *Bacterial strains and growth conditions*

All *Shigella* strains used in this work are listed in Table I. The bacterial samples were isolated from stools before any antibiotic therapy was initiated, during the years of 1980 to 1984, as previously described (Leal *et al.*, 1988a,b). Six *S. flexneri* and four *S. sonnei* samples were recovered from stools of interned diarrhoeic children in two hospitals in Recife (Leal *et al.*, 1988a). Three *S. flexneri* and two *S. sonnei* strains were isolated from stools of nondiarrhoeic children living in low income communities in the surroundings of Recife (Leal *et al.*, 1988b). *Shigella* strains were stored in sealed blood agar base (Difco Laboratories, Detroit, USA) slants at 4°C. Cultures were prepared in YT medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, pH 7.4) and grown at 37°C with aeration.

Table I - *Shigella* strains used in this work.

Strains	Source	Isolation (year)	Reference
<i>S. flexneri</i> 17	healthy child	1980	Leal <i>et al.</i> , 1988b
<i>S. flexneri</i> 152	healthy child	1980	Leal <i>et al.</i> , 1988b
<i>S. flexneri</i> 235	healthy child	1981	Leal <i>et al.</i> , 1988b
<i>S. flexneri</i> 65	diarrhoeic child	1983	Leal <i>et al.</i> , 1988a
<i>S. flexneri</i> 105	diarrhoeic child	1984	Leal <i>et al.</i> , 1988a
<i>S. flexneri</i> 128	diarrhoeic child	1983	Leal <i>et al.</i> , 1988a
<i>S. flexneri</i> 145	diarrhoeic child	1983	Leal <i>et al.</i> , 1988a
<i>S. flexneri</i> 157	diarrhoeic child	1980	Leal <i>et al.</i> , 1988a
<i>S. flexneri</i> 516	diarrhoeic child	1981	Leal <i>et al.</i> , 1988a
<i>S. sonnei</i> 633	healthy child	1981	Leal <i>et al.</i> , 1988b
<i>S. sonnei</i> 807	healthy child	1982	Leal <i>et al.</i> , 1988b
<i>S. sonnei</i> 21	diarrhoeic child	1984	Leal <i>et al.</i> , 1988a
<i>S. sonnei</i> 43	diarrhoeic child	1984	Leal <i>et al.</i> , 1988a
<i>S. sonnei</i> 379	diarrhoeic child	1981	Leal <i>et al.</i> , 1988a
<i>S. sonnei</i> 876	diarrhoeic child	1982	Leal <i>et al.</i> , 1988a

### Antibiotic resistance

Antibiotic resistance patterns of all *Shigella* strains were determined by the method of Kirby and Bauer (Bauer *et al.*, 1966). Discs containing chloramphenicol (30 µg), nitrofurantoin (300 µg), nalidixic acid (30 µg), gentamicin (10 µg), ampicillin (10 µg), trimethoprim (5 µg), carbenicillin (100 µg), sisomicin (10 µg), fosfomicin (50 µg), tetracycline (30 µg) and sulfatrin (25 µg) were assayed in Mueller-Hilton agar (Difco Laboratories, Detroit, U.S.A.).

### Colicin production

Colicin production in *Shigella* isolates was determined by the agar overlay method with the colicin sensitive *Escherichia coli* 22R80 strain (Ozeki *et al.*, 1962). Identification of colicin E1-like producing clones was obtained with the indicator strain BZB2104 (Pugsley, 1985).

### *Virulence assay*

Virulence assays were carried out with the Sereny test in guinea pigs (Sereny, 1955). Infected animals were followed for five days and those strains able to generate kerato-conjunctivitis were considered positive.

### *Congo red binding*

Pigment binding ability of the strains was assayed in tryptic soy broth containing 0.02% Congo Red (Difco Laboratories, Detroit, U.S.A.). The plates were incubated at 37°C for 18 hours to differentiate between pigmented and non-pigmented colonies.

### *Isolation of plasmid DNA*

Plasmid DNA was isolated by the SDS-alkaline lysis method (Birnboim and Doly, 1979). Plasmid molecular weights were calculated with reference *Escherichia coli* strains containing standard plasmids. The *E. coli* 22R80 strain, harboring four plasmids (98, 42, 23.9 and 4.6 MDal), and the *E. coli* EV517 strain, harboring eight plasmids (32, 5.2, 3.5, 3.0, 2.2, 1.7, 1.5, 1.0 MDal) were obtained at the Central Public Health Laboratory (Colindale, England).

### *Agarose gel electrophoresis*

Plasmid DNA was electrophoresed in 12 cm long horizontal agarose gels (1%) with Tris-phosphate running buffer at 100 V during three hours. After the runs, gels were stained with ethidium bromide and visualized under UV illumination.

## RESULTS

### *Phenotypic characterization of the Shigella strains*

All *Shigella* strains isolated from asymptomatic and hospitalized diarrhoeic children were investigated for the ability to invade cells (Sereny test), production of colicin E1, binding of Congo Red and resistance to antibiotics. Table II shows that most of the *Shigella* strains were resistant to only one antibiotic (tetracycline). Three *S. flexneri* and two *S. sonnei* strains harboring multiple antibiotic resistance showed distinct patterns ranging from two to five resistance markers (Table II). Three *S.*

*flexneri* and one *S. sonnei* isolated from diarrhoeic children were resistant to trimethoprim. Ability to bind Congo Red was expressed by only two *S. flexneri* strains recovered from hospitalized children (strains 105 and 128) which were also proficient in generating conjunctivitis in the Sereny test. Strain 128 was also the only *S. flexneri* isolate able to produce colicin E1. On the other hand, all *S. sonnei* strains were able to bind Congo Red and invade epithelial cells. Except for *S. sonnei* strain 807, which produced a distinct colicin, all *S. sonnei* isolates were colicin E1 producers (Table II).

Table II - Characterization of *Shigella* strains isolated from asymptomatic and diarrhoeic children.

Strain		Serotype	Sereny	Col <sup>a</sup>	Pgm <sup>b</sup>	Antibiotic <sup>c</sup>
<i>S. flexneri</i>	17	1	-	-	-	Tet
<i>S. flexneri</i>	152	4a	-	-	-	Tet
<i>S. flexneri</i>	235	2	-	-	+	-
<i>S. flexneri</i>	65	2	-	-	-	Tet, Tm
<i>S. flexneri</i>	105	2	+	-	+	Tet, Tm, Su, Cb, Ap
<i>S. flexneri</i>	128	2	+	+	+	Tet, Tm, Su
<i>S. flexneri</i>	145	3	-	-	-	Tet
<i>S. flexneri</i>	157	2	-	-	-	Tet
<i>S. flexneri</i>	516	2	-	-	+	Tet
<i>S. sonnei</i>	633	1	+	+	+	Tet
<i>S. sonnei</i>	807	1	+	- *	+	Tet
<i>S. sonnei</i>	21	1	+	+	+	Tet, Su
<i>S. sonnei</i>	43	1	+	+	+	Tet, Tm
<i>S. sonnei</i>	379	1	+	+	+	Tet
<i>S. sonnei</i>	876	1	+	+	+	Tet

<sup>a</sup> Ability to synthesize colicin E1, (\*) clone 807 produced a colicin not related to colicin E1.

<sup>b</sup> Binding of Congo Red.

<sup>c</sup> Resistance to tetracycline (Tet); trimethoprim (Tm); carbenicillin (Cb), sulfatrin (Su); ampicillin (Ap).

### Plasmid analysis

Table III shows the plasmid content of all *Shigella* strains analyzed. Most of the strains harbored a heterogeneous population of plasmids with an average number

of 3.8 plasmids per strain and with Mr ranging from 1.0 to more than 100 MDal. Some additional weak DNA bands, representing open circular forms, were sometimes visualized in the agarose gels (Figures 1 and 2). No significant difference could be observed in the number of plasmids in *S. flexneri* strains isolated from asymptomatic carriers and diarrhoeic children. However, *S. sonnei* strains not associated with dysenteric cases harbored significantly less plasmids (2.5 plasmids per strain) than strains recovered from sick children (average of 5 plasmids per strain). A 2.7 MDal plasmid was found in all but one (235 strain) *S. flexneri* isolates. Similarly, two plasmids of 1.2 MDal and 3.4 MDal were isolated from 4 of six *S. sonnei* strains analyzed.

Table III - Number and molecular weight of plasmids found in the *Shigella* strains analyzed.

Strains		Plasmids (MDal)
<i>S. flexneri</i>	17	1.0, 1.3, 1.7, 2.7, 18
<i>S. flexneri</i>	152	2.7, 4.0, 4.5, 6.0, 9.0, 13.0, > 98
<i>S. flexneri</i>	235	1.7, > 98
<i>S. flexneri</i>	65	1.7, 2.7
<i>S. flexneri</i>	105	1.7, 2.7, 50, > 98
<i>S. flexneri</i>	128	1.7, 2.7, 3.0, 4.5, 5.4, > 98
<i>S. flexneri</i>	145	1.7, 2.7, 3.5, 4.5, 4.8, 5.4
<i>S. flexneri</i>	157	1.7, 2.7, 3.5
<i>S. flexneri</i>	516	1.7, 2.7, > 98
<i>S. sonnei</i>	633	3.4, 25, > 98
<i>S. sonnei</i>	807	1.2, 2.1
<i>S. sonnei</i>	21	1.2, 1.5, 3.4, 7.8, > 98
<i>S. sonnei</i>	43	3.4, 4.0, 7.8, 9.2, > 98
<i>S. sonnei</i>	379	1.1, 1.2, 2.1, 3.4, 6.4, 7.8, > 98
<i>S. sonnei</i>	876	1.2, 1.4, 2.8, > 98

High molecular weight plasmids, usually associated with ability to invade mammalian cells and binding of exogenous pigments were isolated from five *S. flexneri* strains and five *S. sonnei* strains (Figures 1 and 2). The high Mr plasmids showed considerable variation in molecular weight and were found in three of the five *Shigella* strains isolated from asymptomatic cases.

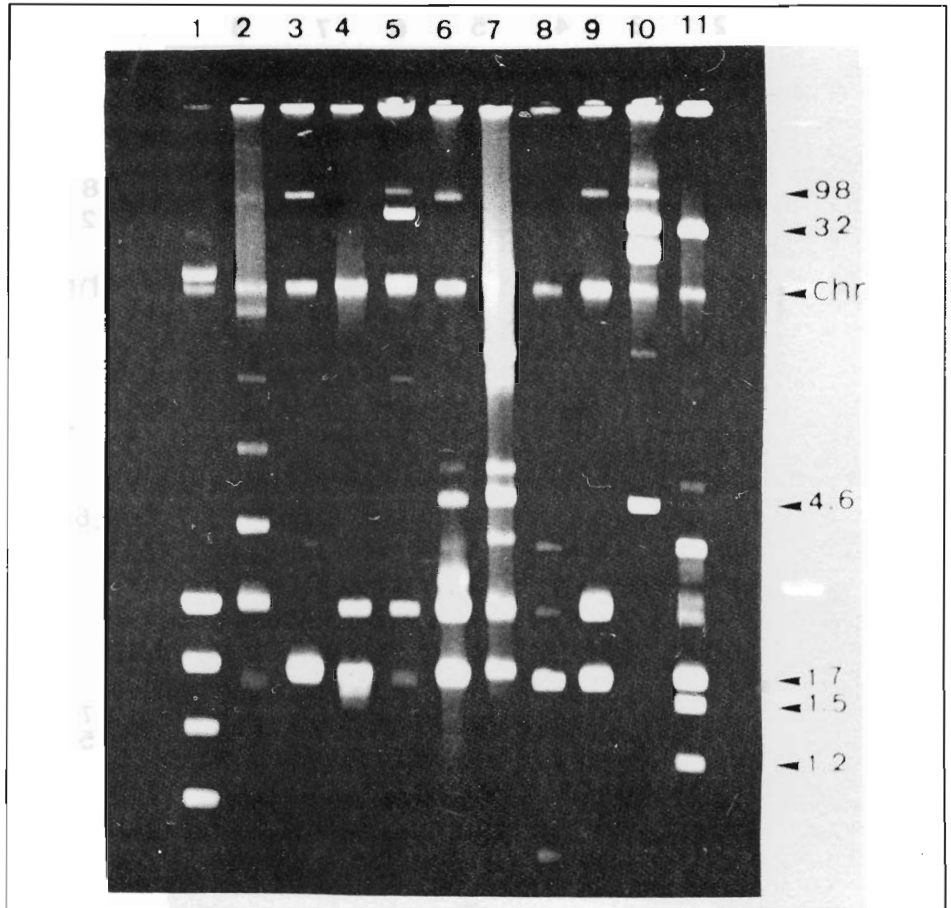


Figure 1 - Agarose gel electrophoresis of plasmid DNA extracted from *S. flexneri* strains. Plasmids were isolated from *S. flexneri* strains: 17 (1), 152 (2), 235 (3), 65 (4), 105 (5), 128 (6), 145 (7), 157 (8), 516 (9); and *E. coli* strains R861 (10) and V517 (11). Molecular weight markers (MDal) are indicated on the right side. The band corresponding to chromosomal DNA is shown. The high molecular weight plasmid of strain 152 is barely visible.

## DISCUSSION

Plasmid fingerprint techniques have been applied previously in the characterization of *Shigella* isolates from different geographical origins. Based on the available plasmid profile data, shigellosis found in underdeveloped countries is

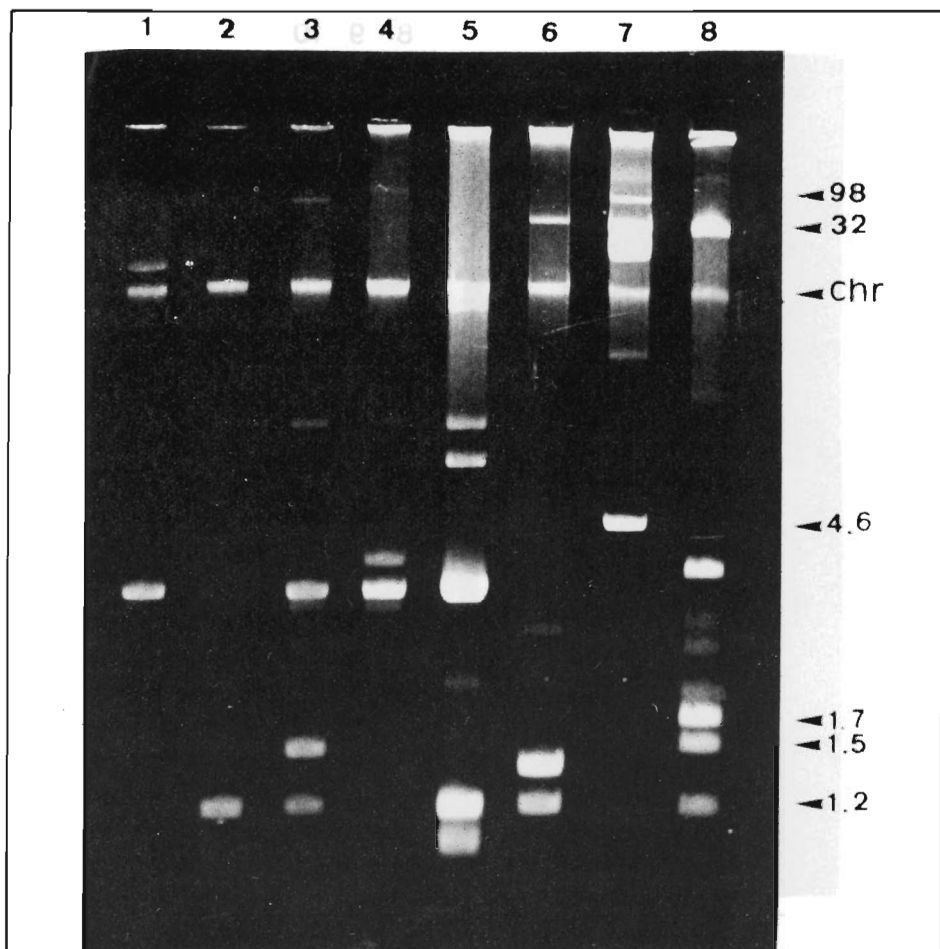


Figure 2 - Agarose gel electrophoresis of plasmid DNA extracted from *S. sonnei* strains. Plasmids were isolated from *S. sonnei* strains: 633 (1), 807 (2), 21 (3), 43 (4), 379 (5), 876 (6); and the *E. coli* strains R861 (7) and V517 (8). Molecular weight markers (MDal) are indicated on the right side. The band corresponding to chromosomal DNA is shown. The high molecular weight plasmids of strains 633 and 379 strains are barely visible.

associated with a diversified set of strains indicating the involvement of many different clones, whereas strains isolated in the developed countries are restricted to one or a few clones accounting for epidemic outbreaks with wide geographical distribution (Tacket *et al.*, 1984; Prado *et al.*, 1987; Mendonza *et al.*, 1988; Haider *et al.*, 1989).

Our present observations were based on 9 *S. flexneri* and 6 *S. sonnei* strains, isolated in Northeast Brazil. The data revealed that nearly all strains had a different set of phenotypic and plasmid profile. The plasmid content of the strains was composed mainly of several molecules with Mr lower than 6 MDal and plasmids with Mr higher than 98 MDal. These data clearly demonstrate that these strains are not genetically related and represent multiple *Shigella* strains in the communities analyzed. This conclusion is in accordance with the fact that the isolates were not associated with a dysenteric outbreak but were recovered from isolated cases in a nonintensive survey during four years (Leal *et al.*, 1988a,b). The establishment of distinct plasmid profiles in strains circulating in these communities can be useful information in the future as an epidemiological tool to trace the spread of one of the pre-existing clones or the import of new strains. Determination of plasmid profiles could also aid considerably in the identification of clones associated with outbreaks originating from a common source.

The genetic involvement of *S. flexneri* and *S. sonnei* plasmids with antibiotic resistance is well documented in the literature (Jamielson *et al.*, 1979; Tonin and Grant, 1987; Bratoeva and John, 1989). Of particular interest was the recent worldwide dispersion of trimethoprim resistance associated with a transposon localized in plasmids of different sizes (Chun *et al.*, 1981; Tiemens *et al.*, 1984; Tonin and Grant, 1987; Bratoeva and John, 1989). In our survey, three *S. flexneri* and one *S. sonnei* strains were resistant to trimethoprim. It is quite probable that the genetic determinants of trimethoprim resistance lie in the high Mr plasmids, since transposons coding for trimethoprim resistance are bigger than 13 kb (Gosti-Testu *et al.*, 1983; Tonin and Grant, 1987). One plasmid which might be involved with the expression of antibiotic resistance in the *S. flexneri* strains is the 2.7 MDal plasmid. This plasmid was found in all *S. flexneri* strains resistant to tetracycline. A similar 2.7 MDal plasmid was also found in *S. flexneri* strains isolated in Bangladesh (Haider *et al.*, 1985, 1989).

Synthesis of colicin E1 by *S. sonnei* was previously associated with a 3.2 MDal plasmid (Sansonetti *et al.*, 1981; Lee *et al.*, 1982). The finding of a 3.4 MDal plasmid in four of the five *S. sonnei* isolates producing colicin E1 supports the possible involvement of this plasmid in the synthesis of this colicin. Therefore, the occurrence of Col E1 plasmids seems to be a frequent feature of *S. sonnei* but not of *S. flexneri* strains isolated in Northeastern Brazil.

High Mr plasmids were found in five *S. flexneri* and five *S. sonnei* strains. The loss of such plasmids in four *S. flexneri* strains was accompanied by the inability to invade cells and bind Congo Red. This result probably indicates that plasmid loss occurred during storage of *S. flexneri* samples, since all strains were invasive when isolated (Leal *et al.*, 1988a,b, and unpublished observations). The fact that three *S. flexneri* strains were not invasive but retained the plasmids might be explained by

genetic rearrangements or mutations in the genes responsible for the control or synthesis of proteins involved with this property. The small differences observed in the Mr of these large plasmids could be evidence for deletions. All six *S. sonnei* strains were Sereny positive and Congo Red positive, but only five harbored high Mr plasmids detected by our experimental conditions. The inability to recover big plasmids from strain 807 could be ascribed to the extraction method used, since their presence would seem to be indicated by the virulence assay.

The present data indicated that *S. flexneri* plasmids were more unstable than those found in *S. sonnei*. Other authors reported contrasting results, which demonstrated a higher instability of *S. sonnei* plasmids in relation to *S. flexneri* (Sansonetti *et al.*, 1981; Sansonetti *et al.*, 1982). An explanation for such results would require the determination of homology between the plasmids of *S. flexneri* strains isolated in Recife and plasmids obtained from different localities.

In a previous work Haider and colleagues (1985) observed that *S. dysenteriae* and *S. flexneri* strains isolated from asymptomatic excretors harbored a smaller number of plasmids than strains isolated from hospitalized patients in Bangladesh. Most of the plasmids detected were small and cryptic, but large plasmids with 120-140 MDal were found in all strains except one (Haider *et al.*, 1985). Based in our data, it was not possible to detect any significant difference in the number of plasmids recovered from *S. flexneri* strains isolated from asymptomatic and diarrhoeic subjects. On the other hand, the two *S. sonnei* strains isolated from asymptomatic patients carried considerably fewer plasmids than the four strains isolated from diarrhoeic patients. The possible relevance of such relationship can only be confirmed after the screening of a statistically significant group of strains.

Based on the present observations, it was not possible to identify clear characteristics or specific plasmids able to distinguish *Shigella* strains isolated from asymptomatic or diarrhoeic subjects. The loss of invasiveness observed in the three *S. flexneri* strains isolated from asymptomatic carriers can be more properly attributed to instability of the genetic determinants during storage than to an inherent feature of these strains. These results corroborate that *Shigella* strains recovered from carriers are genetically similar to strains isolated from diarrhoeic patients.

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## RESUMO

Novo amostras de *S. flexneri* e seis de *S. sonnei* foram isoladas de crianças assintomáticas (3 *S. flexneri* e 2 *S. sonnei*) e diarreicas em Recife. Todas as linhagens foram analisadas e comparadas quanto a invasibilidade (teste de Sereny), produção de colicinas, ligação ao corante vermelho Congo e resistência a antibióticos. As amostras albergavam de dois a sete diferentes plasmídeos e a maioria deles apresentou peso molecular inferior a 6 MDal. As amostras de *S. sonnei* isoladas de portadores assintomáticos continham consideravelmente menos plasmídeos que as linhagens isoladas de pacientes diarreicos. A possível associação entre plasmídeos e resistência a antibióticos, produção de colicina E1 e invasibilidade é discutida.

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